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<b>(21) International Application Number:</b> PCT/SE98/00387 <b>(22) International Filing Date:</b> 4 March 1998 (04.03.98)  <b>(30) Priority Data:</b> 9700769-4      4 March 1997 (04.03.97)      SE  <b>(71) Applicant (for all designated States except US):</b> AMERSHAM PHARMACIA BIOTECH AB [SE/SE]; S-751 84 Uppsala (SE).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> BERGSTRÖM, Jan [SE/SE]; Rönjningsvägen 3, S-740 22 Bälinge (SE). BERGLUND, Rolf [SE/SE]; Rabarbergatan 23, S-754 49 Uppsala (SE). SÖDERBERG, Lennart [SE/SE]; Krusen- berg, Ekshagarna, S-755 98 Uppsala (SE).  <b>(74) Agents:</b> BERGANDER, Håkan et al.; Pharmacia Upjohn AB, Patent Dept., S-751 82 Uppsala (SE).		<b>(81) Designated States:</b> AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> MATRICES FOR SEPARATION AND SEPARATION EXPLOITING SAID MATRICES  <b>(57) Abstract</b>  A matrix comprising a core showing a system of micropores and a surface in which the micropore system has openings. The characterising feature is that the surface is coated with a polymer (I) having such a large molecular weight that it cannot penetrate into the micropores. The matrix can be particulate or in the shape of a monolith. Use of the matrix to separate components in a liquid medium, preferably aqueous, via partition to the matrix. The separation may be based on affinity or on the size and shape of the components. Batchwise as well as chromatographic procedures are comprised.		

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## MATRICES FOR SEPARATION AND SEPARATION EXPLOITING SAID MATRICES

### Technical field

The present invention relates to matrices which can be used for separation of one or more components in a mixture of components. There are also other uses of the particular type of matrices. The separation means that a liquid containing the component(s) is contacted with the particular type of matrix, wherein the component(s) to be removed is (are) partitioned to the matrix and thereby separated from the remaining components which are differently partitioned to the matrix. By the expression "partitioned to the matrix" is meant that components bind or otherwise are adsorbed on (in) the matrix.

The separation methodology may be in form of chromatography on a monolithic matrix or on a packed or fluidized bed of particles or as a batchwise process with suspended particles. Partition to the matrix may be based on affinity or molecular size/molecular shape, such as in affinity and gelchromatography, respectively.

By separation is also meant desalting, buffer exchange, concentration and the like, wherein a separation matrix is contacted with a liquid containing something to be removed.

In the liquid containing the components to be separated, a convective mass transport occurs because of streaming or turbulence. In relation to this mass transport, matrices used for separation according to the above typically show two kind of environments: 1) an environment in which convective mass transport occurs (convective environment) and 2) an environment in which only diffusive transport occurs (diffusion environment). The two environments usually are in contact with each other via the liquid used through openings preventing convective mass transport.

Monolithic matrices and particulate matrices may show both types of environments. For monolithic matrices in the form of through flow pores

functional groups giving different types of physical/chemical interactions with the substances to be purified. In practice, different interactions often counteract each other leading to an impaired result (for example a combination of electrostatic and hydrophobic interaction).

5 Ion exchange on particulate ion exchangers often leads to aggregation of the matrix particles in the presence of macromolecules of opposite charge compared to the particles. These problems are due to surface charges and are accentuated in batchwise suspension procedures and fluidized beds. In this context, there is especially mentioned cell culture  
10 supernatants and other sample solutions containing whole cells and/or parts thereof, including microorganisms of different types.

In the present invention, it has been possible to achieve co-operation of different separation principles on the same chromatographic medium and in this way reduce the number of necessary separation steps in a  
15 purification process.

#### Known matrices

Porous particles of cross-linked agarose coated with dextran are marketed by Amersham Pharmacia Biotech AB (Uppsala, Sweden) under  
20 the designation Superdex®. In the production of Superdex®, dextran is used having a molecular weight distribution which allows the dextran to be present in interior as well as in exterior parts of the particles.

Pore surfaces in membranes/matrices can be coated with polymers of such molecular weight that clogging of the pores is prevented (EP-A-  
25 221046, Monsanto).

Multifunctional matrices of a different construction than the invention have been described previously. See US-A-454485 (Purdue University; Hagestam & Pinkerton), US-A-5522994 (Cornell Res. Found.; & Svec), WO-A-9409063 (Cornell Res. Found.; Frechet & Svec), WO-A-  
30 9408686 (Cornell Res. Found.; Frechet, Smigol & Svec) och WO-A-9317055 (Cornell Res. Found.; Frechet & Hosoya).

Interesting micropores are in several cases smaller than 1  $\mu\text{m}$ , but can also be larger, depending on the intended use of the finished matrix. The micropores correspond in many cases to diffusion pores. The outer surface can be a surface to which convective mass transport can occur.

- 5 Insofar the outer surface corresponds to pore surfaces, the corresponding pores are called macropores below.

By the expression "such molecular weight and shape that it cannot penetrate into the micropores" is meant that the polymer preparation (polymer I), being exploited for coating, has a molecular weight distribution of such a kind that all or substantially all polymer molecules in the preparation are excluded from transport into the micropores, when the preparation is dissolved in a liquid which, via the micropores, can be transported into the naked matrix. This means that polymer I, when it is anchored to the outer surface, can give separation characteristics of the surface, which are different from the separation characteristics of the micropores.

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#### Naked matrix (the core)

As a rule, basic matrices for chromatography and molecular sieves are suitable as cores in the invention. Such basic matrices may be monolithic or particulate, based on organic or inorganic material, based on one or more polymers, be hydrophilic, hydrophobic or intramediate hydrophilic etc.

20

Examples of hydrophilic organic cores are polymers showing several hydrophilic groups, such as hydroxy groups, (-OH), amine groups (primary, secondary, tertiary, quaternary amine/ammonium), carboxy groups (-COOH/-COO<sup>-</sup>), repeating groups -OCH<sub>2</sub>CH<sub>2</sub>- and -OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>-, -OCH<sub>2</sub>CH<sub>2</sub>(CH<sub>3</sub>)-, amide groups (-CONH<sub>2</sub> possibly substituted with lower alkyl (C<sub>1-10</sub>) etc., wherein hydrophilic groups preferably are directly bound to single monomer units in the polymer. Polyhydroxy polymers and polyamides are typical hydrophilic polymers,

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under the trade name Sepharose® and Superdex®, respectively, cross-linked dextran in bead shape, for example qualities marketed under the trade name Sephadex®, cellulose, for example qualities marketed under the trade name Sephacel®, cross-linked porous particles of polyacrylamide derivatized with dextran in the pores, for example Sephacryl®, and monodisperse and polydisperse porous particles of for example styrene-divinyl benzene polymer which have been hydrophilized, for example qualities marketed under the trade name MonoBeads® and Source®. These trade marks correspond to products sold by Amersham Pharmacia Biotech AB, Uppsala, Sweden.

Polymer in surface layer delimiting micropores (polymer I).

Polymer I may either be hydrophilic, hydrophobic or intermediately hydrophilic depending on which liquid medium is to be used for the separation.

According to the invention, it is often suitable that surfaces in the pores of the core, the liquid medium and polymer I have similar hydrophilic/hydrophobic characteristics to simplify the penetration (wetting) of the inner and outer surfaces of the matrix by the liquid media. For the combination of polar liquid media, for example aqueous, with polar cores suitable as polymer I are: water soluble forms of polyvinylalcohol (PVA), dextran, cellulose, agarose, polyethers, such as polyethyleneoxide, polypropyleneoxides and their co-polymers etc. For the combination of less polar or non-polar liquid media and cores, polystyrene, hydrophobized dextran or similarly treated cellulose may be suitable as polymer I. This does not exclude the use of a combination of hydrophobic surfaces in the micropores, delimiting surface layers with hydrophilic or less non-polar polymer I and hydrophilic liquids.

Polymer I may be functionalized before it is attached on the surface delimiting the micropores. Functionalisation in this context means that polymer I is provided with the groups, present on the finished surface

interval 1-10,000  $\mu\text{m}$ , preferably 1-5,000  $\mu\text{m}$  and most preferably 1-1,000  $\mu\text{m}$ . The particles of the population may also be monodisperse having a particle size within any of these interval.

Macropores of monolithic matrices may be of a different size and shape analogous to the above discussion about particles. In such embodiments the surface of the macropores is covered by polymer I.

#### Density of particulate matrices

Depending on the use of the finished matrix, it may exist a need for matrices having a larger, a lesser or the same density as the liquid medium in which they are intended to be used (density for matrix saturated with the liquid medium). Production of particulate matrices can be done by incorporating filling-agents, often in the form of smaller particles, in the matrices. Particulate matrices having a density deviating from the liquid medium used for separation have mostly been used for batchwise suspension procedures and for expanded/fluidized beds. Typically for this embodiments the density is over 1.02 g/cm<sup>3</sup>, preferably over 1.1 g/cm<sup>3</sup>, or under 0.98 g/cm<sup>3</sup>. See, e.g. WO-A-9200799 (Kerm-En-Tek/ Upfront Chromatography). See also WO-A-9118237 (Amersham Pharmacia Biotech AB) which also describes that large practical advantages are achieved if the used fluidized bed only is stabilized by a liquid flow. It has also been popular to use magnetic filling-agents to simplify separation of the particles and stabilisation of fluidized beds by magnetic fields.

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#### Production of the matrices - coating with polymer I

The production typically involves contacting a porous core according to the above with a liquid, which in dissolved form contains polymer I having a size and shape preventing the polymer to penetrate into micropores of a predetermined diameter. For micropore systems with large pores, polymer I can be in the form of colloidal particles. Polymer I, the

sent International Patent Application). Following the coating reaction, remaining alkene groups may be activated for incorporation of a functionality and/or cross-linking in the remaining part of the core, either in the form of layers or in the whole core. The method enables production  
5 of multifunctional chromatographic media, in which each functionality is located to a layer in the core.

### Functionalisation of the matrices

The ready matrix may contain functional groups (ligands) of the same  
10 kind as those used within liquid chromatography. Examples are:

1. ion exchange groups
2. bioaffinity groups
3. hydrophobic groups
4. groups that can be used for covalent chromatography
- 15 5. sulphur-containing groups, for example, for so called thio-philic interaction,
6. chelate or chelating groups,
7. groups with aromatic systems giving rise to so called  $\pi$ - $\pi$ -interaction with different compounds,
- 20 8. groups giving hydrogen bonds
9. hydrophilic groups etc.

The substitution degree for at least one ligand from the groups 1-9 in the micropores is often different from the substitution degree for the same ligand in the surface layer delimiting the micropores. In many  
25 embodiments of the matrices of the invention, the substitution degree for a ligand in the surface layer is zero or close to zero, at the same time as the same ligand is present in the micropore system. Also the reverse may be at hand. Ligands chosen from the groups 1-2 and 4-8 may be absent in the surface layer.

30 Ion exchanging groups can be anion exchanging, such as primary, secondary, tertiary, quaternary ammonium group, sulphonium group

ammonium group (primary, secondary or tertiary) with a hydroxy group at a distance of 2 or 3 carbons from the ammonium nitrogen.

Hydrophilic groups according to the invention are mainly single hydroxy, lower hydroxy alkyl with one or more hydroxy groups, groups  
5 containing repeating structures -  $\text{CH}_2\text{CH}_2\text{O}$ - etc. The groups often are of low molecular weight, for example, with less than 25 carbon atoms.

Hydrophilic or hydrophobic polymer groups with or without ligands can give gel filtration characteristics. The polymers of this type of group may be cross-linked.

10 Suitable groups (ligands) are typically coupled to the matrix via a bridge which may have a structure selected according to known techniques. The bridge structure may be polymeric, for example hydrophilic or hydrophobic polymer, having one or more of the groups 1-10 according to the above on each linker. Common bridge names have been  
15 "spacers", "tentacles", "extenders", "fluff", "linkers" etc., each of which sometimes has a certain meaning. Hydrophobic bridges are mainly suited for hydrophobic liquid media and, if they are polymeric, often lead to better availability and capacity for introduced groups 1-9. The corresponding is true for hydrophilic bridges in combination with hy-  
20 drophilic liquid media. Examples of hydrophilic polymer bridges are polysaccharides such as dextran and other water-soluble polyhydroxy polymers. Polymer bridges can be used to create matrices with gel filtration characteristics.

## 25 Utility

The main utility of the matrices is separation of the kind mentioned in the introduction. The mentioned exclusion limits and functional groups enable use of the matrices according to the invention for separation of nucleic acid, proteins including peptides and other organic and  
30 inorganic compounds. The separation may be performed from mixtures containing similar or very different components, everything from single



A. Production of cross-linked allylated agarose in particle shape  
Cross-linked agarose (34  $\mu\text{m}$  particles) produced by a reaction between  
epichlorohydrin and agarose in the presence of NaOH according to Po-  
rath et al. (J. Chromatog. 60 (1971) 167-77 and US-A-3959251) was  
5 reacted with allylglycidyl ether with NaOH as a base to an allyl level  
( $\text{CH}_2=\text{CHCH}_2\text{OCH}_2\text{CHOHCH}_2-$ ) of 0.21 mmole/mL gel. In aqueous media  
we have succeeded to produce gels with an allyl level in the interval  
0.005-0.450 mmole/mL gel. If the reaction is performed in organic sol-  
vents even higher substitution degrees can be obtained.

10 B. Dissolving of raw dextran. Raw dextran is a non-hydrolyzed dex-  
tran from *Leuconostoc mesenteroides* with ultrahigh molecular weight,  
often between 10-30 million Dalton. Following purification with re-  
peated ethanol precipitations, raw dextran is in principle completely free  
from low molecular sugar compounds. The raw dextran that was used  
15 was from Amersham Pharmacia Biotech's production plant in Staffan-  
storp, Sweden. 47.5 g ethanol precipitated and freeze dried raw dextran  
was dissolved in 230 mL distilled  $\text{H}_2\text{O}$  in a 1,000 mL three-necked flask  
under slow stirring.

C. Bromination of cross-linked allylated agarose. 200 mL drained  
20 allylated agarose produced according to step A, 200 mL distilled water  
and 5.72 g NaOAc were added to a 1,000 mL three-necked flask.  
Thereafter, bromination occurs by dripping in an excess of bromine  
water. Subsequent to bromination the gel is washed on a glass filter  
with distilled water.

25 D. Coupling reaction: The gel from step C is aspirated dry and  
transferred to the flask containing dissolved raw dextran from step B  
during careful stirring. The mixture is allowed to equilibrate for one  
hour. Thereafter the reaction is started by addition of 29.2 g NaOH and  
0.73 g  $\text{NaBH}_4$  dissolved in 91 mL distilled  $\text{H}_2\text{O}$ . The temperature is set  
30 to  $50^\circ\text{C}$  and the reaction is allowed to proceed over night (for example  
16 h) and then the reaction is stopped by neutralisation with concen-

C. Bromination of allylated Phenyl Sepharose HP: 250 mL allylated Phenyl Sepharose HP produced in step A, 250 mL distilled water and 5.23 g NaOAc (anhydrous) are added to a 1,000 mL three-necked round flask. Thereafter, an excess of elementary bromine is added by dripping to achieve bromination, and then the gel is washed on a glass filter with distilled water.

D. Coupling reaction: The gel from step C is aspirated dry and transferred to the flask containing dissolved raw dextran from step B. The mixture is allowed to equilibrate with careful stirring. Thereafter the reaction is started by addition of 36.5 g NaOH and 0.95 g NaBH<sub>4</sub> dissolved in 114 mL of distilled water. The temperature is set to 35°C and the reaction is allowed to proceed over night (16 h) under careful stirring. The reaction is then stopped by pouring off the reaction mixture in a 2 litre beaker and neutralisation with concentrated HOAc to pH <7, preferably 5-6. Finally the gel is washed with distilled water on a glass filter.

#### Exemple 4: Q Sepharose HP coated with high molecular dextran

A. Production of allylated Q substituted cross-linked agarose (allylated Q Sepharose HP): Q Sepharose HP is commercially available from Amersham Pharmacia Biotech AB. It consists of cross-linked agarose (30 µm particles) which is substituted with quaternary groups (CH<sub>3</sub>)<sub>3</sub>N<sup>+</sup>CH<sub>2</sub>CHOHCH<sub>2</sub>- with ion exchanging capacity of 0.15-0.20 mmole/mL gel). Allylation occurs by reacting the finished particle with allylglycidyl ether with NaOH as a base to an allylic level (CH<sub>2</sub>=CHCH<sub>2</sub>OCH<sub>2</sub>CHOHCH<sub>2</sub>-) of 0.088 mmole/mL.

B. Dissolving of raw dextran: 4.28 g of ethanol precipitated and freeze dried raw dextran according to the above are dissolved in 26.0 mL distilled water in a 100 mL three-necked flask under slow stirring.

C. Bromination of allylated Q Sepharose HP: 20 mL Q Sepharose HP produced in step A (substitution degree allyl groups 0.088

The selectivity is markedly changed when modification is performed with raw dextran. The selectivity curve,  $K_d = f[\log(\text{molecular weight})]$  becomes steeper and is considerably biased against lower molecular weights. The exclusion limit decreases to just below 100,000 daltons.

- 5 Cross-linking of the bound raw dextran contributes to further biasing of the selectivity curve towards lower molecular weights,  $K_d$  values decrease further.

Example 6: Ion exchange, raw dextran modified Q-Sepharose HP  
 10 Q-Sepharose HP (Amersham Pharmacia Biotech AB) modified according to example 4 was packed in HR 5/5 columns from Amersham Pharmacia Biotech AB to a bed height of about 5 cm. Gradient chromatography was performed on a FPLC system provided with measuring cells for UV<sub>280</sub> and conductivity with the following conditions:

15	Buffer A)	20 mM Tris-HCl pH 8.2
	Buffer B)	20 mM Tris-HCl pH 8.2 + 0.5 M NaCl
	Flow rate	1.0 mL/min.
	Gradient volume	20 mL
	Samples	Thyroglobulin, Ferritin, IgG, CO-Hb, EGF and in
20		certain cases transferrin
	Sample conc.	About 1 mg/mL in buffer A
	Sample volume	50 $\mu$ L

The elution concentration at peak maximum was determined for unmodified Q-Sepharose HP and Q-Sepharose HP modified with raw dextran. A dimension-less value on the lock-effect was calculated as the  
 25 quotient between the elution concentrations for Q-Sepharose HP modified with raw dextran and unmodified Q-Sepharose HP, respectively. This quotient is a measure of the ability of the sample to penetrate the outer raw dextran layer and interact with the inner of the bead, the  
 30 lock-effect. The value 0 means that the sample is completely excluded and that no ionic interaction occurs. The value 1.0 is obtained when the

Buffer A)	10 mM H <sub>3</sub> PO <sub>4</sub> -KOH and 2.0 M NH <sub>4</sub> SO <sub>4</sub> pH 7.0
Buffer B)	10 mM H <sub>3</sub> PO <sub>4</sub> -KOH pH 7.0
Flow rate	1.0 mL/min.
Gradient volume	20 mL
5 Samples	Thyroglobulin 660 kD, ferritin 443 kD, IgG 152 kD, chymotrypsinogen A 25 kD, lysozyme 14.3 kD, EGF 6 kD,
Sample conc.	1 mg/mL except for EGF 0.16 mg/mL
Sample volume	50 $\mu$ L

10 From figure 3, it appears that the adsorption and elution performance on raw dextran modified Phenyl Sepharose HP markedly deviates from the unmodified Phenyl Sepharose HP. The largest deviations are obtained for larger proteins. Large molecules, such as IgG are excluded, from the hydrophobic phenyl groups present in the interior of the beads  
15 and are directly eluted without adhering to the matrix. Small molecules like EGF, lysozyme and chymotrypsinogen A enter into the particles and are adsorbed. IgG and ferritin provide two peaks probably due to that the samples also contained low molecular weight material.

Non-used killed coupling groups (for example CH<sub>2</sub>OH-CHOHCH<sub>2</sub>-) in  
20 the interior of the matrix provide an exclusion effect, which decreases the availability of the phenyl groups and affects the elution of the molecules which are adsorbed in the interior of the bead.

Fig. 1. Gel filtration,  $K_d = f[\log(\text{molecular weight})]$  for Sepharose HP  
25 modified with raw dextran without and with a subsequent cross-linking.

Fig. 2. Ion exchange, lock-effect =  $f[\log(\text{molecular weight})]$  Q-Sepharose HP modified with raw dextran.

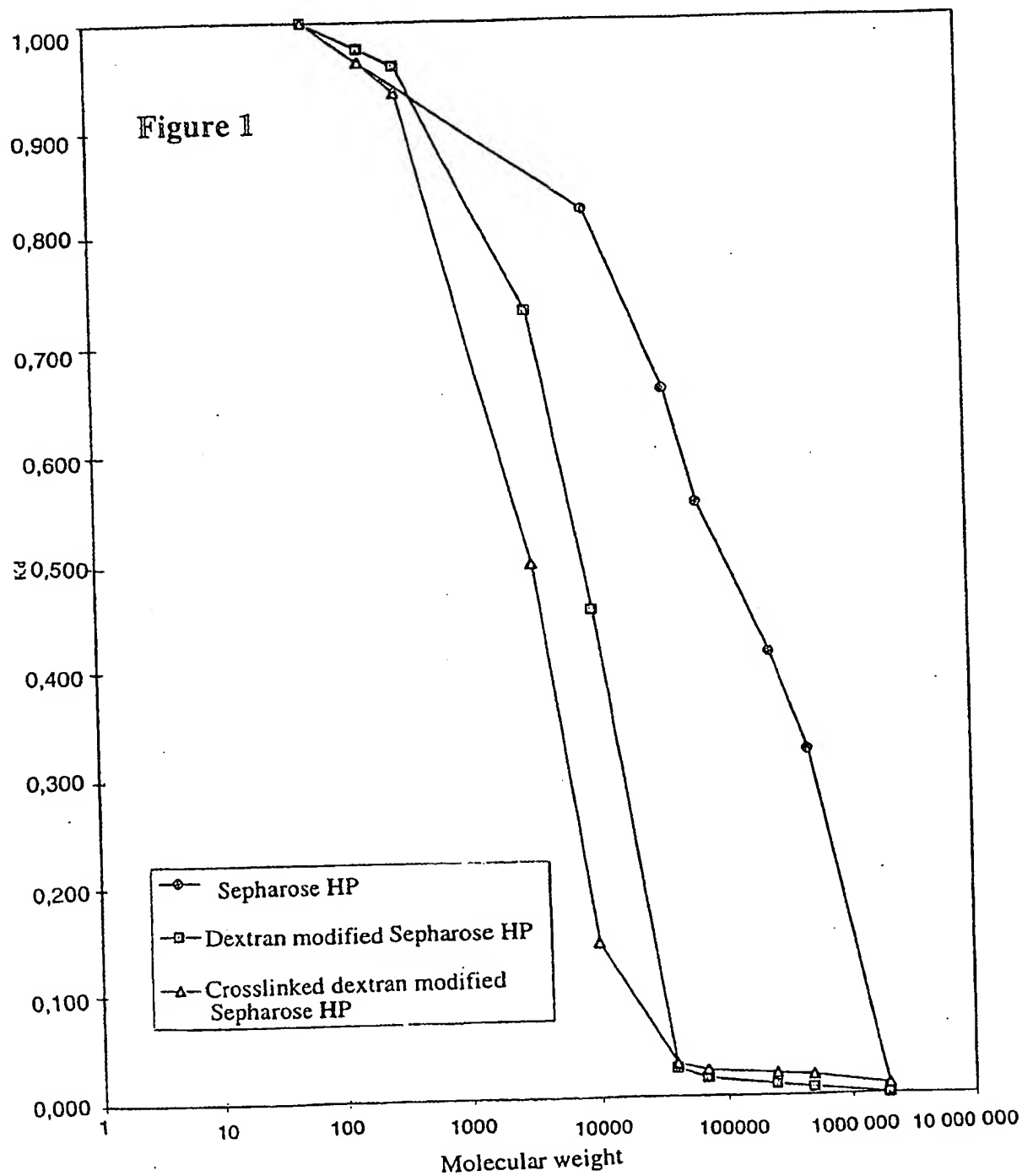
## CLAIMS

1. A matrix comprising a core showing a micropore system and a surface in which the pore system has openings, characterized in that the surface is covered with a polymer (I) having such a large molecular weight that it cannot penetrate into the micropore system.  
5
2. A matrix according to claim 1, characterized in that the diameter of the openings is less than 1  $\mu\text{m}$ .  
10
3. A matrix according to any of the claims 1-2, characterized in that the micropore system represents a diffusive pore system with openings preventing convective mass transport into the system.  
15
4. A matrix according to any of the claims 1-3, characterized in that the surface in the micropores and polymer I are hydrophilic.
- 20 5. A matrix according to any of the claims 1-4, characterized in that the surface in the micropores show hydroxy groups.
6. A matrix according to any of the claims 1-5, characterized in that polymer I has been cross-linked and/or covalently bound to the surface in which the micropore system has openings.  
25
7. A matrix according to any of the claims 1-5, characterized in that the core is built of a polymer (II).
- 30 8. A matrix according to claim 7, characterized in that polymer II is hydrophobic and that its surfaces have been hydrophilized.

- c. hydrophobic groups
  - d. groups which can be exploited for covalent chromatography
  - f. chelate or chelating groups,
  - 5 g. groups with aromatic systems which can be exploited for so called  $\pi$ - $\pi$ -interaction,
  - h. hydrogen binding groups
  - i. hydrophilic groups.
15. A matrix according to claim 14, characterized in that the substitution degree for at least one of the at least one selected ligands in  
10 the micropores is different from the substitution degree for the same ligand in the surface layer which is built of polymer I.
16. A matrix according to any of the claims 14-15, characterized in  
15 that the substitution degree for at least one of the at least one selected ligands in the micropores or in polymer I is substantially 0.
17. A matrix according to any of the claims 1-16, characterized in  
20 that it is in the form of a set of particles whose particle size is in the interval 1-10,000  $\mu$ m.
18. A matrix according to claim 17, characterized in that when the  
particles are filled with a liquid medium, they have a density which  
is distinct from that of the liquid medium.
- 25
19. A matrix according to any of the claims 17-18, characterized in  
that it is monodisperse.
20. A matrix according to any of the claims 17-18, characterized in  
30 that the particles represent sizes and/or densities within an interval.

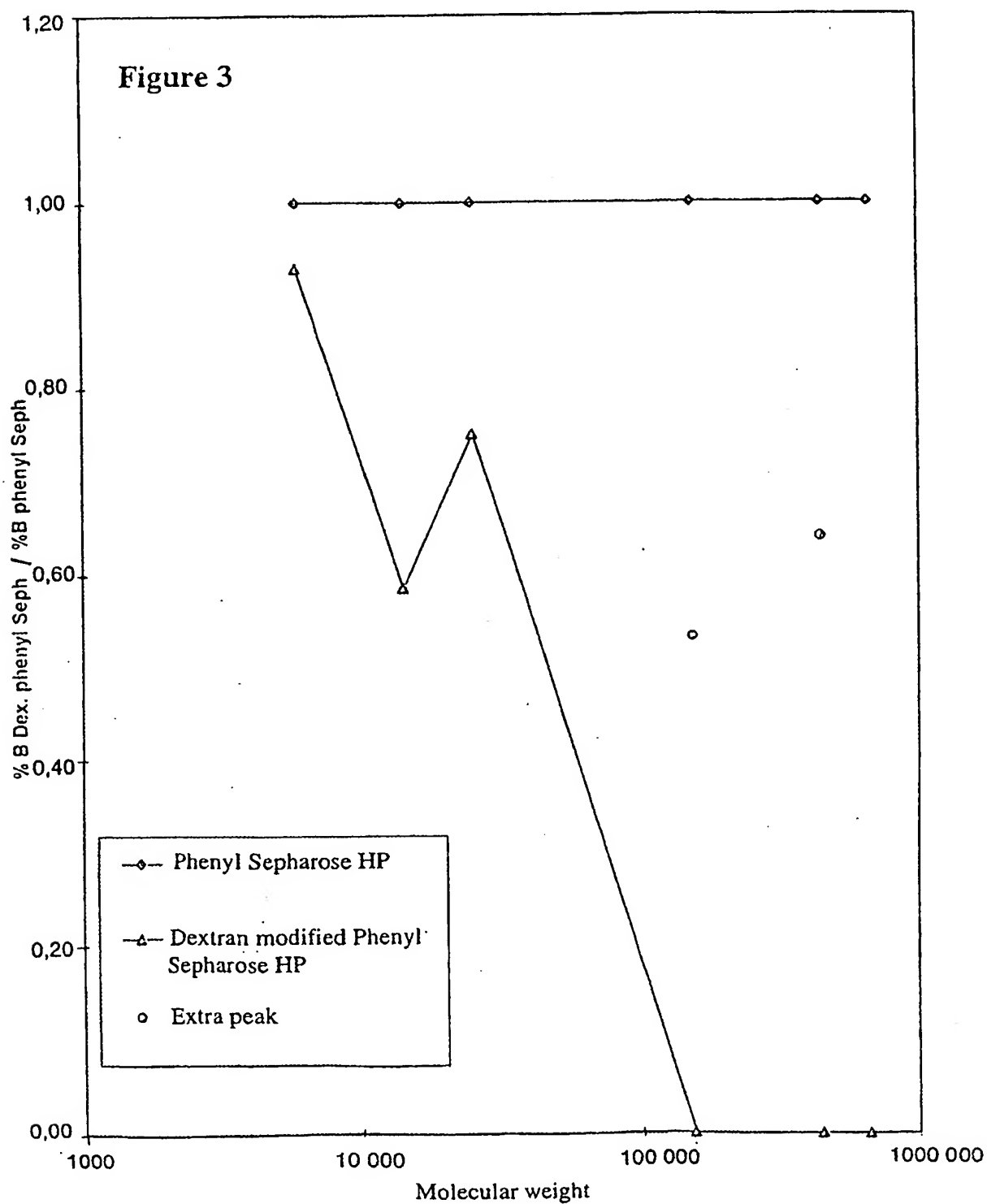
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The effect of a lock of raw dextran on Sepharose HP  
Kd as a function of the molecular weight for various dextrans



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The effect of a lock of raw dextran on Phenyl Sepharose HP  
Potassium phosphate buffer 10 mM pH 7.0  
Ammonium sulphate 2.0-0 M





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Information on patent family members

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